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Small-Molecule Allosteric Activation of Human Glucokinase in the Absence of Glucose

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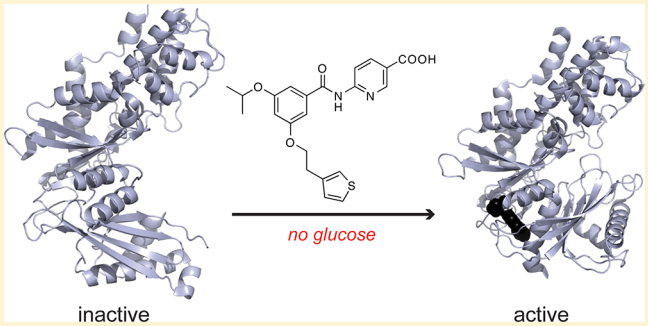
Supporting Information

ABSTRACT: Synthetic allosteric activators of human glucokinase are receiving considerable attention as potential diabetes therapeutic agents. Although their mechanism of action is not fully understood, structural studies suggest that activator association requires prior formation of a binary enzyme−glucose complex. Here, we demonstrate that three previously described activators associate with glucokinase in a glucose-independent fashion. Transient-state kinetic assays reveal a lag in enzyme progress curves that is systematically reduced when the enzyme is preincubated with activators. Isothermal titration calorimetry demonstrates that activator binding is enthalpically driven for all three compounds, whereas the entropic changes accompanying activator binding can be favorable or unfavorable. Viscosity variation experiments indicate that the k_{cat} value of glucokinase is almost fully limited by product release, both in the presence and absence of activators, suggesting that activators impact a step preceding product release. The observation of glucose-independent allosteric activation of glucokinase has important implications for the refinement of future diabetes therapeutics and for the mechanism of kinetic cooperativity of mammalian glucokinase.



KEYWORDS: *Maturity onset diabetes of the young type II, glucokinase activators, kinetic cooperativity, hysteretic enzyme*

Human glucokinase (GCK, hexokinase IV) catalyzes the ATP-dependent phosphorylation of glucose in the first step of glycolysis.[1] GCK is expressed in pancreatic β-cells where it functions as a sensor for glucose-dependent insulin secretion.[2] The unique kinetic features of GCK are directly linked to its functional role in maintaining glucose homeostasis. GCK displays mild positive cooperativity toward glucose and a $K_{0.5}$ value that approximates physiological glucose concentrations.[3] The kinetic cooperativity of human GCK is mechanistically interesting because the enzyme is a functional monomer and contains only one binding site for glucose.[4,5] The physiological importance of GCK is underscored by the observation that mutations in the *glk* gene have a profound impact on blood glucose levels. Inactivating *glk* mutations have been directly linked to maturity onset diabetes of the young type 2 (MODY-II), while mutations producing hyperactive GCK variants cause persistent hyperinsulinemic hypoglycemia of infancy (PHHI).[6]

As a result of its central role in glucose homeostasis, GCK has emerged as an attractive target for diabetes therapeutic development. In 2003, investigators from Hoffmann-La Roche described the identification and in vivo characterization of a lead compound for diabetes treatment that targeted GCK.[7] Functional analysis revealed that this molecule operates as a mixed-type allosteric activator, increasing the enzyme's maximal velocity, decreasing the $K_{0.5}$ value for glucose, and reducing the kinetic cooperativity of the enzyme. This GCK activator yielded promising results in terms of therapeutic potential, enhancing both hepatic glucose metabolism and glucose-stimulated insulin release from isolated rat islets. In vivo tests with diabetic rodent models substantiated these results, showing reduced blood glucose levels and augmented plasma insulin upon activator treatment. To date, a variety of structurally unique allosteric activators of GCK have been identified.[8−11] One such molecule, piragliatin (RO4389620), successfully advanced through phase II clinical trials for the treatment of type 2 diabetes; however, these trials were halted for undisclosed reasons.[12,13]

Structural studies indicate that GCK activators bind to an allosteric site on the enzyme that is ∼20 Å removed from the glucose binding site.[14] Interestingly, the binding site for allosteric activators overlaps with the location of many single-site variants identified in patients with PHHI.[15] Indeed, the kinetic consequences of PHHI-associated mutations are identical to those observed when the enzyme is assayed in the presence of activator.[4] The allosteric binding site is noticeably absent in the crystal structure of unliganded GCK, suggesting that small-molecule activation is dependent upon glucose association. Consistent with this postulate, Ralph et al.

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failed to detect binding of activators to the unliganded enzyme using differential scanning calorimetry and a scintillation proximity assay.[16] Moreover, a 2010 status update of GCK activators suggested that these potential therapeutic agents exclusively target the binary enzyme−glucose complex.[12] In contrast to these reports, one recent study supports the ability of synthetic activators to associate with the unliganded enzyme.[17] To probe the mechanism of action of GCK activators in more depth, we functionally characterized the interaction of three previously described therapeutic lead agents in the presence and absence of glucose.[14,16−18] Our results provide clear evidence supporting the ability of all three GCK activators to function via a glucose-independent mechanism.

We synthesized GKA22 with an overall yield of 9% using a six-step procedure adapted from a previous report (Figure S1, Supporting Information).[18] Details of the synthetic procedure, as well as a description of the relevant intermediates, are provided in Supporting Information. Two other synthetic activators, Compound A and RO-28-1675, were commercially obtained. To investigate the glucose dependency of activator association, we conducted a series of experiments in which activators were pre-equilibrated with the enzyme in the absence of glucose. Previously, Neet and co-workers observed a lag in enzyme progress curves when assays were conducted in the presence of 30% glycerol. This lag was eliminated upon preincubation with glucose, which was interpreted in terms of a slow, glucose-induced conformational transition.[19] We hypothesized that if activators could associate with GCK in the absence of glucose, a similar decrease in the lag would be observed. To test this postulate, the activating properties of each compound were evaluated using a standard assay for GCK activity linking glucose 6-phosphate production to the reduction of $NADP^+$ via the action of glucose 6-phosphate dehydrogenase. To exclude glycerol from assay mixtures, which might promote activator association,[11] we utilized a stopped-flow apparatus to monitor early time points in the assay. Reactions were initiated via the addition of 1 mM glucose and monitored until the steady-state velocity was attained. Consistent with previous reports, assay mixtures containing only GCK produced progress curves showing a lag in the attainment of steady-state; however, in the presence of activators, we observed a reproducible decrease in the lag (Figure 1). To quantify the lag for each condition, curves were fitted to a single-exponential decay of one enzyme state to another.[19] Extrapolation of lifetime measurements from the fits produced the transition time (τ), which reports on the extent of the delay in reaching the steady-state regime. In the absence of activator, the lag was characterized by a τ value of 0.51 min (Table 1). When GCK was preincubated with GKA22 (20 μM), the τ value was reduced to 0.30 min. Compound A and RO-28-1675 (20 μM) produced similar effects, yielding transition times of 0.34 and 0.35 min, respectively. Notably, at concentrations near the solubility limits of these compounds (25−100 μM), we did not observe complete elimination of the lag. It is unclear whether this failure is due to an inability to saturate the enzyme with activator at these concentrations or whether the activators are unable to fully shift the equilibrium toward the more active species. Nevertheless, our results demonstrate that activator association promotes an active enzyme conformation, similar to the effects produced by glucose. Importantly, this perturbation occurs without prior glucose association, in the absence of glycerol. The ability of activators to reduce the lag indicates that unliganded GCK populates at least one conformational state capable of associating with activators.

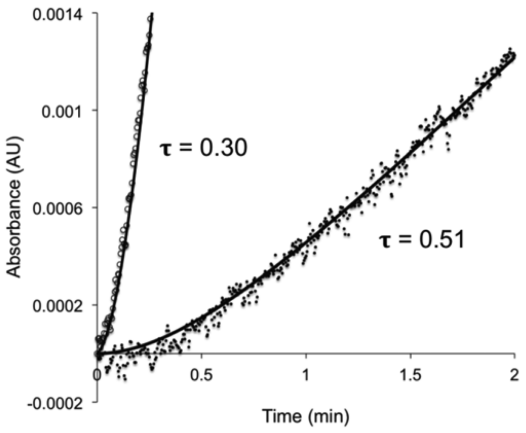
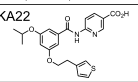
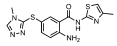
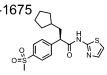


Figure 1. Lag in the transient-state GCK progress curve obtained in the absence of activator (filled circles) is reduced in the presence of GKA-22 (open circles).

Table 1. Thermodynamic and Kinetic Characterization of GCK Activators at 25°C

Effector	n	K_d (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	TΔS (kcal/mol)	τ (min)
None (control)	-	-	-	-	-	0.51 ± 0.11
GKA22 	0.90 ± 0.02	0.17 ± 0.04	-9.2 ± 0.1	-17.0 ± 0.5	-7.7 ± 0.4	0.30 ± 0.01
Cpd A 	0.94 ± 0.01	0.44 ± 0.06	-8.7 ± 0.1	-13.0 ± 0.2	-4.3 ± 0.3	0.34 ± 0.01
RO-28-1675 	1.0 ± 0.10[a]	0.50 ± 0.20[a]	-8.6 ± 0.6[a]	-6.2 ± 0.5[a]	2.4 ± 0.4[a]	0.35 ± 0.02

[a] Data obtained from ref. 21

To fully characterize the enzyme−activator interaction, we investigated the thermodynamics of activator association using isothermal titration calorimetry (ITC). In the absence of glucose, we did not detect activator binding at concentrations approaching the solubility limits of these compounds. This failure likely results from an inability to sufficiently populate the GCK−activator complex at the activator concentrations (≤110 μM) and activator/enzyme ratios (≤10:1) possible in these studies. By comparison, the activator/enzyme ratio used in the transient-state assays described above equaled 2000:1. In the presence of saturating glucose concentrations, association of GKA22 and compound A with GCK is accompanied by a large, favorable change in enthalpy and an unfavorable entropic component (Figure 2, Table 1). Negative values for these parameters indicate a binding event dominated by specific interactions.[20] In contrast, a previous study found that interaction of the binary GCK−glucose complex with RO-28-1675 and another synthetic activator involves favorable entropic changes.[21] Glucose binding to unliganded GCK is also accompanied by a highly favorable entropic component ($T\Delta S$ = 10.7 kcal/mol).[22] Structural studies provide no obvious rationale for the differences in the entropic consequences of activator association, although the differential extent to which solvent is excluded from the allosteric binding pocket could provide one explanation.[23−29] Docking studies are consistent

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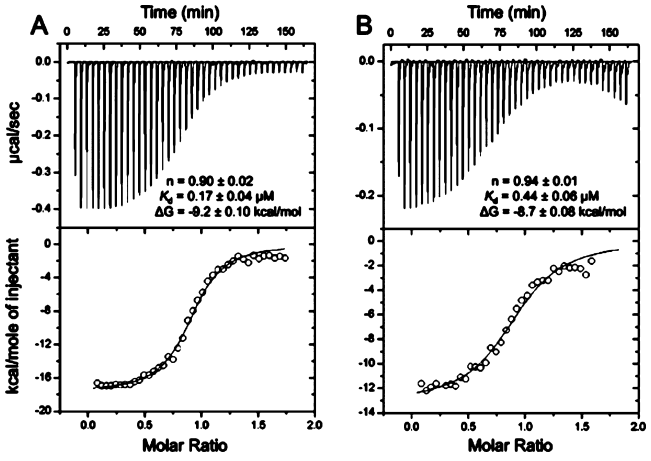

Figure 2. (A) Representative isotherm for GKA22 (110 μM) binding to glucokinase (11 μM) in the presence of glucose. (B) Representative isotherm for CpdA (100 μM) binding to glucokinase (10 μM) in the presence of glucose. Thermodynamic parameters represent the average of two or more independent experiments.

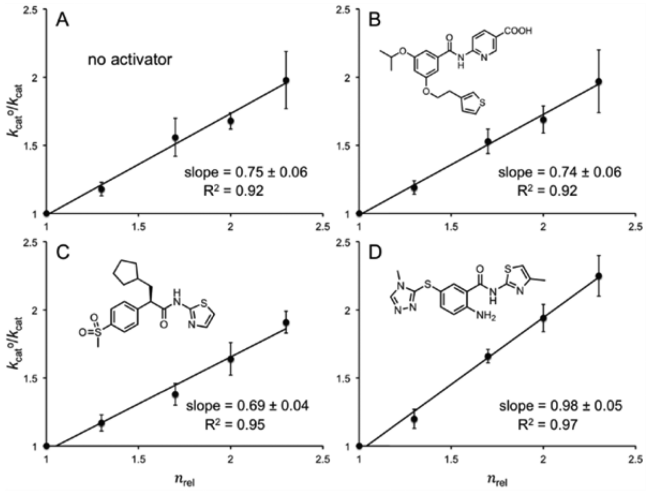

Figure 3. Normalized values of k_{cat} as a function of relative solvent viscosity in the absence (A) or presence of GKA-22 (B), RO-28-1675 (C), and CpdA (D). A slope of unity is consistent with product release being fully rate-limiting.

with this possibility (Supporting Information). Despite differences in the thermodynamic details of activator association, the ability of each activator to produce comparable reductions in the lag indicates that activators elicit a similar kinetic effect.

All synthetic activators described to date have the ability to reduce the glucose $K_{0.5}$ value. A subset of these compounds has the added stimulatory attribute of increasing V_{max}.[7−11] Two of the activators examined in this study, RO-28-1675 and compound A, increase k_{cat} by 30% and 10%, respectively. GKA22 leaves k_{cat} unaffected. As a first step in understanding these differences, we used viscosity variation to probe the extent to which the k_{cat} value was limited by a diffusion-limited process, such as product release, in the absence and presence of activators.[30] Using sucrose as a microviscogen, we conducted GCK assays under saturating substrate conditions. A plot of the normalized value of k_{cat} as a function of relative solvent viscosity in the absence of activator revealed that the k_{cat} value is fractionally dependent upon solvent viscosity, with a slope of 0.75 (Figure 3A). By comparison, we observed that the k_{cat} value of a truncated variant of GCK, for which chemistry is expected to be fully rate-limiting,[31] was independent of solvent viscosity. These observations are consistent with the rate of product release contributing substantially to the value of k_{cat} for unactivated GCK. In the presence of GKA22, which has no measurable impact upon V_{max}, the viscosity dependence of k_{cat} is unchanged (Figure 3B). Activation by RO-28-1675, which produces the largest increase in V_{max}, also results in no measurable change in the dependence of k_{cat} upon solvent viscosity (Figure 3C). If the increase in V_{max} caused by RO-28-1675 results from an enhanced rate of product release, one would expect the dependency upon solvent viscosity, as reflected by the slope, to decrease. That activation by RO-28-1675 does not alter the dependence of k_{cat} upon relative viscosity suggests that the increase in V_{max} caused by this compound involves acceleration of a step preceding product release. Interestingly, compound A functions in a similar manner, as it also fails to decrease the dependency of k_{cat} upon solvent viscosity. Instead, compound A appears to accelerate an earlier step(s) in the catalytic cycle to such an extent that it no longer contributes to the value of k_{cat}. Thus, upon activation with compound A, product release becomes fully rate-limiting, as reflected by a slope of unity (Figure 3D). Together our data suggest that activators such as RO-28-1675 and compound A, which increase the maximal velocity of GCK, do so by enhancing the rate of a step preceding product release. Moreover, the magnitude of the slope of the normalized value of k_{cat} as a function of relative viscosity for unactivated GCK suggests that the largest increase in V_{max} that can be expected from mechanistically similar activators is ∼30%. If larger increases in the maximal velocity of GCK are a desirable attribute of a diabetes therapeutic agent, an activator that accelerates the rate of product release is needed.

The findings presented herein provide insight into the mechanism of kinetic cooperativity in monomeric human glucokinase. Current models suggest that GCK cooperativity results from a slow, glucose-induced conformational reorganization of enzyme structure that takes place during the catalytic cycle.[32−34] One model for cooperativity, the ligand-induced slow transition mechanism, posits a conformational selection mode of ligand binding.[32] Another cooperativity model, the mnemonic mechanism, implies an induced fit mode of glucose association.[35] Although our present results do not allow us to definitively distinguish between these two possibilities, the ability of activators to associate with GCK in the absence of glucose is consistent with the enzyme existing as a conformational ensemble whose population is shifted upon ligand binding. In principle, any ligand or binding partner capable of shifting the conformational equilibrium of unliganded GCK in favor of the active state would function as an activator.

Our discovery that activators can function in a glucose-independent fashion may also have implications for the development of glucokinase-targeted therapeutic agents for the treatment of type 2 diabetes. Clinical trials of one GCK activator, piragliatin, were discontinued following phase II for undisclosed reasons.[12,13] One potential concern with this and other activators is that these drugs might be too effective at promoting glucose utilization, which could lead to hypoglycemia.[36] In light of our findings, it seems possible that long-term administration of activators could lead to an enzyme that is permanently primed for rapid glucose turnover. The physiological consequences of such a condition are unknown, especially in terms of GCK's ability to associate with several

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known binding partners including the glucokinase regulatory protein and the pro-apoptotic protein BAD.[11] The impact of glucose-independent activation of GCK in the liver, another prominent tissue where this enzyme is localized, is also unclear. These uncertainties emphasize the need for continued investigations into the mechanism of action of these important putative therapeutic agents.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures and experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GCK, glucokinase; MODY-II, maturity onset diabetes of the young type 2; PHHI, persistent hyperinsulinemic hypoglycemia of infancy; $NADP^+$, nicotinamide adenine dinucleotide phosphate; ITC, isothermal titration calorimetry

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